Studies on the Pathogenesis of Atherosclerosis

I. Adhesion and Emigration of Mononuclear Cells in the Aorta of Hypercholesterolemic Rats

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In rats with diet-induced hypercholesterolemia, two concomitant changes began to occur within 1 week and persisted for 1 year: an increase in total plasma cholesterol and an increase in the number of mononuclear cells adhering to the aortic intima (up to values 50 times normal). Adherent cells were ~90% monocytes and ~10% lymphocytes. Adhesion was focal, with some preference for ostia of aortic branches; it was followed by migration into the subendothelial space. The subendothelial monocytes/macrophages progressively became foam cells, thus giving rise to microscopic "fatty streaks." Ultimately, typical atherosclerotic plaques were formed. Four possible mechanisms of increased cell adhesion are suggested. Endothelial changes were mild; myelin figures arising from the endothelial surface were seen by electron microscopy. Endothelial denudation was never observed, neither in light-microscopic preparations stained with $AgNO_3$ nor by ultrastructure. Platelet participation was minimal. It is concluded that in this model atherosclerotic plaques are initiated by mononuclear cell adhesion and emigration; endothelial denudation is not a necessary step in their pathogenesis. (Am J Pathol 1983, 113:341–358)

THE ARTERIAL INTIMA—in "normal" laboratory animals—has a peculiar feature which has never been adequately explained: small numbers of mononuclear cells adhere here and there to the endothelium, singly or in small clusters, and sometimes penetrate into the subendothelial space. This phenomenon, which suggests a minimal pathologic event, has been described by light as well as by electron microscopy[1–4]; we reported earlier that macrophages and lymphocytes occasionally adhere to and then penetrate into the aortic intima of normal rats.[3]

Although it is not known why mononuclear cells behave in this manner, it is well established that both adhesion and penetration are greatly exaggerated in hyperlipidemic animals and in early human atherosclerosis, to the point of suggesting a comparison with diapedesis in an inflammatory reaction.[1,2] Still and O'Neal, in 1962, were the first to publish electron micrographs of macrophages "clinging to and apparently penetrating" into the aortic intima of rats fed an excess of butter.[5] Similar findings were then reported from many sources; the cells were variously referred to as macrophages or monocytes,[6–8] leukocytes,[9] monocytes, lipophages or foam cells,[10] monocytes,[11–15] monocytes and lymphocytes.[4,16] These studies concern hyperlipidemic mammals, but similar observations were made on hyperlipidemic birds[17] as well as on nonhyperlipidemic but hypertensive mammals.[18–22] A few reports mention also the focal accumulation of granulocytes,[23,24] including "mast cells-basophils"[1] and eosinophils.[25] The "stickiness" of endothelium for lymphocytes[26–28] and granulocytes[29,30] was observed also in tissue cultures.

In most of the papers mentioned, the phenomenon of mononuclear cell adhesion and transendothelial passage was reported incidentally. An exception is represented by the studies of Gerrity et al,[11–15] which

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focused on the role of blood monocytes as precursors of foam cells in atherogenesis. In these experiments, carried out on pigs, adhesion occurred within 2 weeks on the diet, and 10 weeks in advance of early intimal lesions.[11] Clowes et al have reported adherent and emigrating mononuclear cells in hypercholesterolemic rats "at 3 months but not before."[7]

It is essential to establish the timing and sequence of mononuclear cell emigration with regard to hyperlipidemia and intimal changes. Blood-derived macrophages are an established component of atherosclerotic plaques.[31-33] If cellular invasion is the first event and leads to plaque formation without intimal denudation (ie, without loss of endothelial lining), the "endothelial injury" theory of atherogenesis[34] becomes difficult to defend. We approached this problem in the hyperlipidemic rat because this model produces—in the aorta—typical atherosclerotic plaques; furthermore, it affords an excellent overview of the aortic endothelium after staining by the "silver lines" method. This first paper will deal primarily with the features, timing, and quantitation of *mononuclear cell adhesion and emigration*; the results are relevant to the pathogenesis of atherosclerosis, because they show that plaques in our model can be initiated by this cellular event, and without visible endothelial loss. A preliminary report of these findings was published earlier.[35]

Materials and Methods

Animals and Diets

The animals used in this study were maintained and used in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals*, prepared by the ILAR, NRC (DHEW Publication NIH 78-23, 1978) and guidelines of the Animal Care Advisory Committee of the University of Massachusetts Medical School. Seventy-six male Wistar rats (Charles River Breeding Laboratories, Wilmington, Mass) weighing 225-250 g were divided into 3 groups and fed the following diets[7] *ad libitum*: 1) Standard Purina Formulab Chow #5008 (controls); 2) Standard Chow with 4% cholesterol and 1% cholic acid (CC diet); 3) Standard Chow with 4% cholesterol, 1% cholic acid, and 0.5% 2-thiouracil (CCT diet). The cholesterol diets were prepared by Bioserv Inc. (Frenchtown, NJ). Every 2 weeks the animals in each group were weighed, and blood was obtained for lipid determinations and lipoprotein electrophoresis.

On the basis of preliminary experiments, the rats were sacrificed within four time intervals: 1-2, 3-6, 7-11, and 12-52 weeks. For each group the following procedures were carried out (Table 1).

Blood Lipid Studies

Blood (1.5 cc) was collected from the severed tail tip into EDTA-coated test tubes and spun at 2000 rpm for 5 minutes. The plasma was drawn off and tested for total cholesterol and triglycerides by an ABA 100 chemical analyzer (Abbott) with results expressed as milligrams per deciliter. Blood samples were obtained from all rats from the baseline period before the onset of the experimental diet and thereafter at biweekly intervals until sacrifice. One-hundred forty-seven determinations were recorded for the cholesterol levels (56 for CC rats, 52 for CCT rats, 39 for controls) and 139 determinations for the triglyceride levels (54 for CC rats, 49 for CCT rats, and 36 for controls).

Lipoprotein Electrophoresis

Lipoprotein separations were done with a Corning Agarose Film Cassette System (470191, Scientific Products, Bedford, Mass). Aliquots of 2 μl plasma were placed in each of the 8 wells of electrophoresis film (Corning 470100), which was then placed into the cell cover; each cell channel was filled with 95 ml of a universal barbital buffer, pH 8.5 (Corning 470180), and electrophoresis was performed for 35 minutes at room temperature. Subsequently the films were carefully blotted dry and incubated at 70 C (Corning 470144) for 20 minutes, stained with Fat Red 7B (Corning 470126) for 2 minutes, destained in 75% methanol, dried again, and photographed.

Table 1—Distribution of Animals Studied by Diets, Stages, and Techniques

	1-2 weeks	3-6 weeks	7-11 weeks	12-52 weeks	Total number of rats studied†
CC					
LM*	4	6	7	10	27
EM	1	3	3	8	
CCT					
LM*	7	4	8	13	32
EM	3	2	2	10	
Control					
LM*	2	4	2	9	17
EM	1	1	2	2	

* LM (light microscopy) includes all specimens studied with silver nitrate, phagocytosis (carbon, nonspecific esterase), oil red O, and hematoxylin stains for morphometry.

† The total number of rats studied corresponds to the number of rats examined by light microscopy. The EM (electron microscopy) specimens were taken from the same animals as for LM.

Light Microscopy

The aorta was prepared for study *en face* as follows. Under ether anesthesia, with the animal supine, a left thoracotomy was performed; the thoracic aorta just beyond the aortic arch was cannulated with a 20-gauge Angiocath (Deseret, Sandy, Utah), and solutions were infused at 110 mm Hg in this sequence: glutaraldehyde 3% (in 0.1 M cacodylate buffer, pH 7.4) for 20 minutes, 0.05% $AgNO_3$ for 1 minute, bromides (CoBr 3%, NH_4Br 1%) for 1 minute. Outflow was through severed jugular veins. Thereafter the abdominal aorta (from the renal to the iliac branches) was excised, immersed in fixative, exposed under a desk lamp with a 60-watt bulb for a total fixation time of 5 hours, transferred to cacodylate buffer, and refrigerated at 4 C overnight. The next morning the vessel—still unopened—was dissected free of loose periadventitial tissue, rinsed in distilled water for 1 minute, immersed in 70% isopropyl alcohol for 10 minutes, rinsed again in distilled H_2O, stained with filtered Harris' hematoxylin (by injection into the lumen) for 45 seconds, and washed again in distilled water. It was then cut into segments 3–5 mm long; these were slit open longitudinally with iridectomy scissors, laid out flat (endothelium face up) on a glass slide, coverslipped, allowed to dry overnight, and mounted with Permount.

Staining with oil red O (10 minutes) was performed on 3–5-mm segments of glutaraldehyde-fixed aorta, followed by rinsing in 70% isopropyl alcohol, then in water, and staining for 1 minute with Harris's hematoxylin. The segment was then opened, mounted flat in glycerine jelly, and examined *en face*.

The *nonspecific esterase* reaction was used for the study of macrophages.[36,37] Dilute Karnovsky's fixative (1% paraformaldehyde and 1.25% glutaraldehyde [high purity] in Sörensen's buffer) was perfused for 10–20 minutes, then washed with 0.2 M sucrose in Sörensen's buffer for 60 minutes. The abdominal aorta was excised, left in buffer overnight at 4 C, then transferred to Sörensen's buffer M/15 and cleaned of periadventitial tissue. Segments 3–5 mm long were incubated in a freshly prepared solution of hexazotized pararosanalin and α-naphthyl acetate at room temperature for 3–10 minutes, rinsed in distilled water, and counterstained with methyl green (0.5%) or hematoxylin for 1 minute; then the specimens were cut open, placed on a glass slide, coverslipped, and studied *en face*.

Phagocytosis by adherent cells was tested by injecting intravenously 3 times (in 10 rats) 0.3 ml of biologic ink (Pelikan, batch c11/1431a, Gunther-Wagner, Hanover, Germany) 140, 80, and 20 minutes before sacrifice.

Paraffin-Embedded Material

Immediately after glutaraldehyde perfusion small specimens of abdominal aorta were postfixed in 10% buffered formaldehyde and processed as usual; the sections were stained with hematoxylin and eosin (H&E) or van Gieson–elastin.

Electron Microscopy

Perfusion fixation was carried out as described above with 3% glutaraldehyde for 20 minutes. Thereafter the abdominal aorta was excised, left in 3% glutaraldehyde for a total fixation time of 5 hours, and immersed in cacodylate buffer overnight. The specimen was then postfixed in 1.3% OsO_4, dehydrated in graded alcohols, and cut transversely into rings or longitudinally into strips. Tissue samples were embedded in Polybed 812 (Polysciences, Inc., Warrington, Pa). One-micron-thick sections were stained with toluidine blue; ultrathin sections were cut with a diamond knife on an LKB Ultrotome III, mounted on copper grids, stained with uranyl acetate and lead

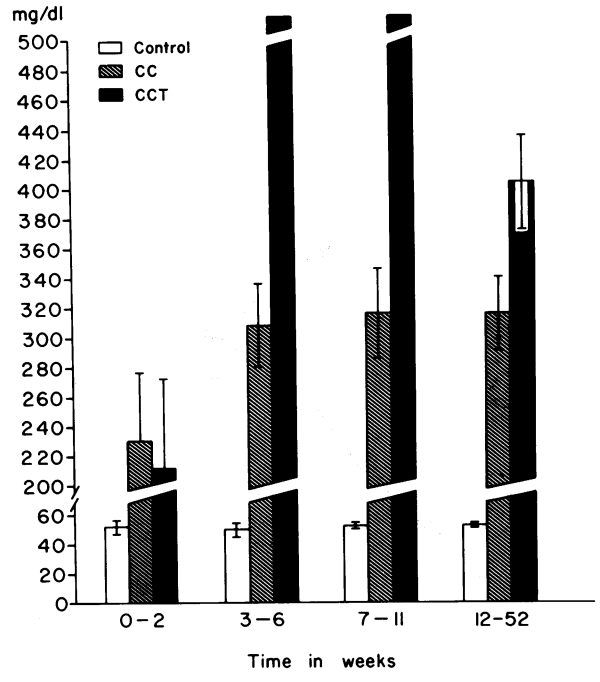


Figure 1—Total plasma cholesterol (average ± SEM) and its changes with time in control and experimental rats. The highest values are reached by the rats on the CCT diet. The difference between experimental and control values was highly significant ($P < 0.001$ for CC and CCT, Student t test).

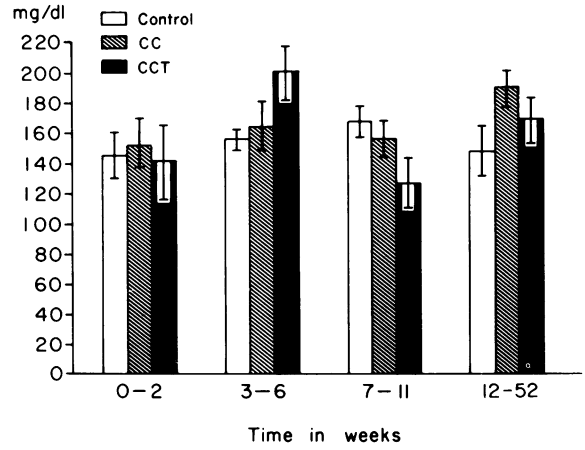


Figure 2—Plasma triglycerides (average ± SEM) as determined over 1 year in all groups of rats.

citrate, coated with carbon, and studied with a Philips 301 electron microscope.

Morphometry

To measure the number of white blood cells adherent to the endothelium, a segment of aorta stained with hematoxylin was scanned *en face* at ×200, and the *total* number of adherent cells was first counted (switching temporarily to ×400 as required to inspect single cells). Then the preparation was photographed as a whole at ×25, and its total surface area was determined planimetrically, using an H1 PAD digitizing tablet (Houston Instrument, Austin, Tex) and a PDP 11/40 computer (Digital Electronic Corp., Maynard, Mass). The total number of adherent cells on a given segment was divided by the total area of that segment in square millimeters, yielding the number of adherent cells per square millimeter.

Results

Control rats and rats on the cholesterol-cholate diet (CC rats) gained weight steadily up to >500 g; rats on the cholesterol-cholate-thiouracil diet (CCT rats) gained little or no weight.

Plasma Lipids

In controls, cholesterol remained at the normal range of 50–55 mg/dl (Figure 1). In the experimental rats, cholesterolemia was already increased by the end of the first week and continued to rise thereafter. In CCT rats, by 3 weeks the value had risen about 10-fold beyond the range of the method (500 mg/dl) and was expressed accordingly; measurements on diluted aliquots showed that values expressed as >500 mg/dl could be as high as 1150 mg/dl with a mean of 900 mg/dl (Figure 1). Differences between control and hypercholesterolemic values were highly significant ($P < 0.001$). Plasma triglyceride values changed but little, and without any significant correlation with cholesterolemia (Figure 2).

Electrophoresis (Figure 3) showed different patterns for the two hypercholesterolemic diets. *In CC rats*, there was a sharp increase in lipoproteins migrating in the pre-beta region (middle band); the alpha band at first decreased, then increased (but still remained decreased in comparison with the controls); the beta band was decreased. *The CCT rats* showed a progressive increase in alpha and beta bands.

At autopsy the first aortic lesions appeared in the CCT rats at 27 weeks, as small, whitish, sharply limited patches. All animals killed after 1 year had grossly visible lesions throughout the entire aorta. No gross lesions were found in the CC rats up to 1 year.

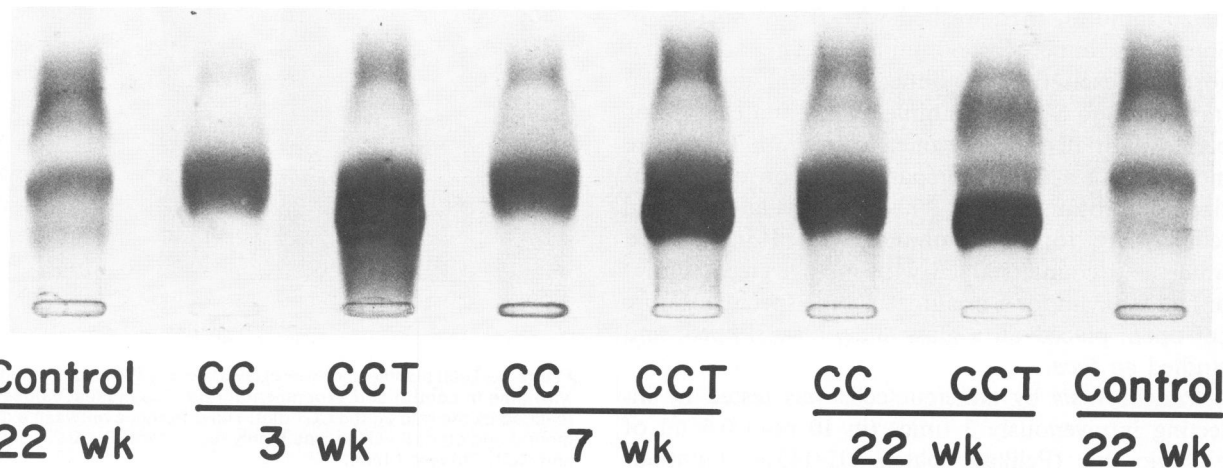


Figure 3—Electrophoretic pattern of plasma lipoproteins from 8 rats. The alpha band is toward the top (see text).

Light Microscopy

Histologic Sections

Paraffin-embedded aortas of rats kept for 1 year on the CCT diet showed different types of intimal thickening. The simplest lesions were small groups of foam cells beneath the endothelium. Larger masses of foam cells were covered not only by endothelium but also by a thin layer of mononucleated cells, some rounded, some elongated (Figure 4); in some cases the mass of foam cells had become necrotic, giving rise to a lipid-rich mass covered by a thin "fibrous cap" (Figure 5). In the adjacent media some smooth muscle cells contained lipid droplets. The superficial layers of the media showed a variable amount of cell loss and necrosis.

Epon-embedded aortas (1 μ thick) were valuable for the study of the atherosclerotic plaques, which were shown in considerable detail and far more clearly than in paraffin-embedded samples (Figures 6 and 7). For the study of adherent cells, however, *en face* preparations were more informative than sections.

In the CCT rats which had been 27 weeks on the diet, the lesions were sharply outlined. Attached to the endothelium were mononucleated cells (Figures 6 and 7). The endothelium itself contained fat droplets; the number and size of the droplets increased with time on the diet. In the intimal thickening two different types of fat-laden cells could be recognized: *macrophages* with a rounded nucleus and many small lipid droplets of equal size (ie, typical foam cells) and elongated *smooth muscle cells*, with an oval nucleus and 1–4 larger lipid inclusions. The difference between the two cell types is obvious (Figures 6 and 7). Many crystals of cholesterol were present, mostly along the internal elastic lamella. Medial damage included fragmentation of the internal elastic lamella as well as of some outer elastic lamellas.

Studies en Face

Qualitative Studies

Silver-treated aortas, surface-stained with hematoxylin, showed the expected normal pattern in controls (Figure 8) and no endothelial loss in hyperlipidemic rats (Figures 9 and 10). A black ring was sometimes visible around an adherent cell, presumably caught in the act of emigrating. The adherent white blood cells stood out clearly against the background of the endothelium, because their nucleus stained much more heavily. Adherent cells of controls were usually single and widely scattered (Figure 8). Hypercholesterolemic animals showed large clusters and swarms (Figures 9 and 10), including as many as hundreds of cells. The phenomenon was always focal; some patches were located around the ostium of an arterial branch (Figure 11); others appeared in seemingly random fashion on the aortic intima and tended to have an elongated shape along the axis of the aorta. Most of the adherent cells had a short tail directed upstream, best visible in the absence of $AgNO_3$ impregnation (Figure 12). It was not uncommon to find a dense cluster of cells—up to 12—attached to the body and margins of a single endothelial cell (Figure 13).

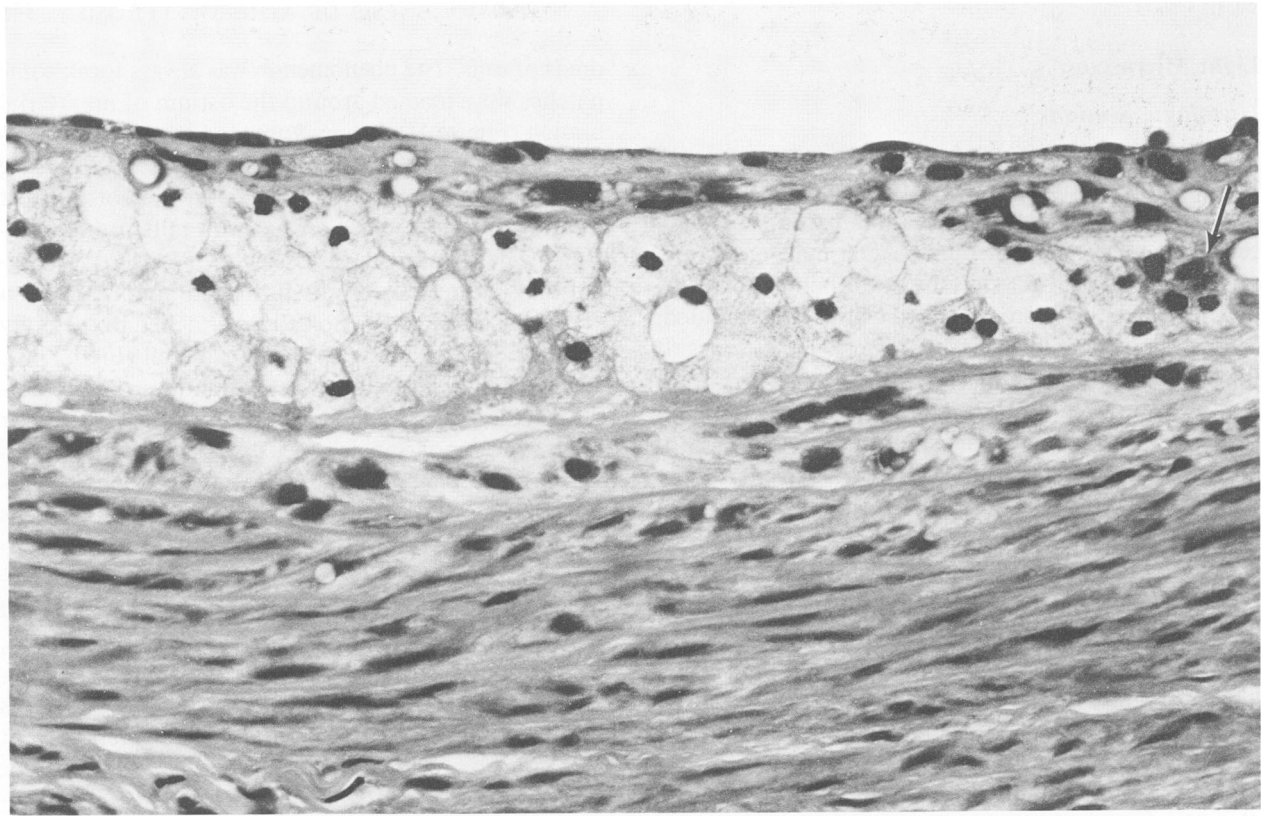
Most adherent cells were mononuclear; polymorphonuclear cells were the rare exception. To further identify the mononuclear cells, we used phagocytosis and nonspecific esterase. In rats overloaded with carbon black intravenously, most of the adherent cells took up carbon particles (massively or in small granules); some always remained carbon-free (this fraction being about 10%). Nonspecific esterase showed that most adherent cells had positive, intensely red, coarse granules; a few had fine, pink grains. Occasional cells were entirely nonreactive. Red blood cells were often present between and around the adherent mononuclear cells; this phenomenon was constant (ie, it could not be attributed to occasional unsatisfactory perfusion) and increased with the duration of hypercholesterolemia.

In addition to adherent cells, *subendothelial cells* were found. They were characterized by a round, pale nucleus which distinguished them from endothelium and from surface-attached cells. In controls, these subendothelial cells were rare and usually single; in hypercholesterolemic rats they became more and more numerous with advancing stages (Figure 10) and progressively acquired the aspect of foam cells. They were concentrated in the same areas where mononuclear cells were attached to the surface. From 27 weeks onward in CCT rats, small, raised plaques were formed, consisting of foam cells, mononuclear cells, and smooth muscle cells, covered with a continuous layer of endothelium. Adherent mononuclear cells were present over and near the plaque (Figures 6 and 7).

Preparations stained with *oil red O* showed endothelial fat droplets already at 1–2 weeks and increasing thereafter, with irregular distribution. In unstained preparations examined with polarized light, some (but not all) of the lipid droplets gave rise to typical Maltese crosses. Foam cells were seen after 8 weeks, both above and below the endothelium.

Quantitative Studies (Figure 14)

In controls, adherent cells were 2–6/sq mm. In both CC and CCT rats there was a 4-fold increase during the first 2 weeks; thereafter, the CC rats showed a 20–



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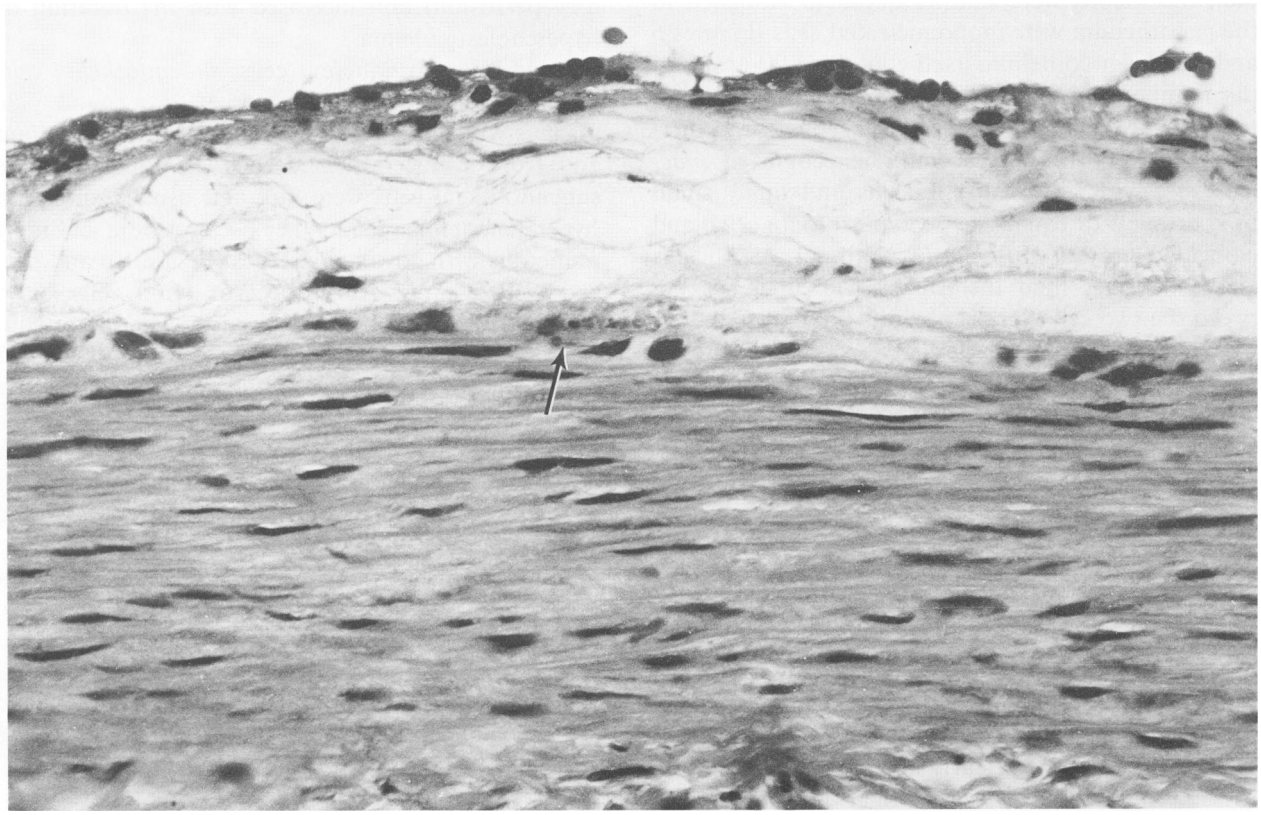

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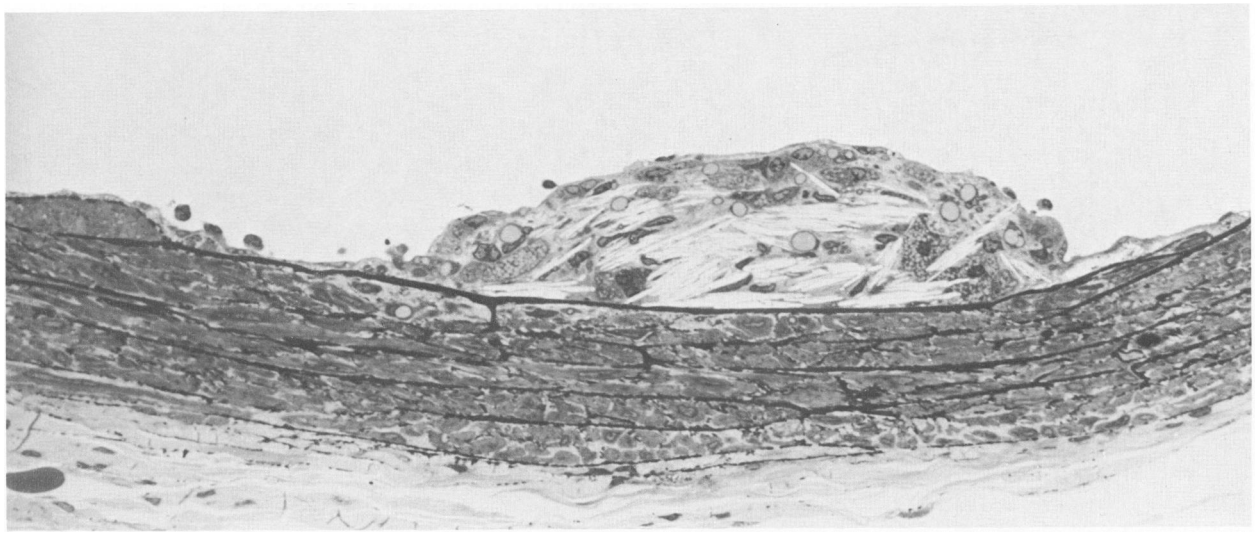
Figures 4 and 5—Two types of plaques in the abdominal aorta of a rat after 1 year on the CCT diet. **Figure 4**—Foam cells laden with small lipid droplets beneath a thin "fibrous cap" consisting of endothelium and 1–2 layers of mononuclear cells, rounded or elongated. The latter are shown by electron microscopy to be smooth muscle cells. Notice the mitosis (*arrow*). In the inner media a couple of smooth muscle cells contain fat droplets. A few cells are attached to the endothelial lining. (H&E, ×5100) **Figure 5**—A similar plaque but presumably more advanced: the mass of foam cells has become necrotic. The media is unremarkable except for a few calcium deposits (*arrow*). (H&E, ×5100)



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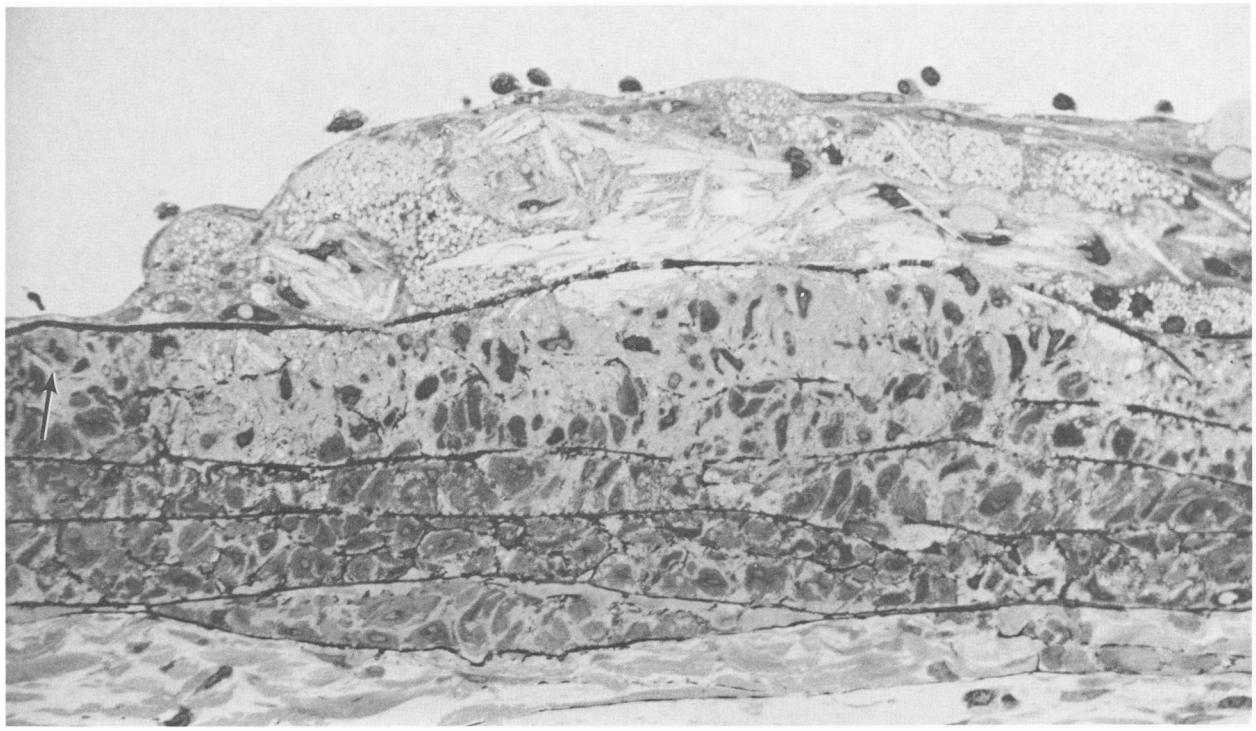

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Figure 6—Abdominal aorta of a rat after 11 months on the CCT diet. A small, well-circumscribed plaque in the intima, containing foam cells, smooth muscle cells (with characteristic large, round lipid droplets), and cholesterol crystals. Notice the cells attached to the endothelial surface. (Epon section, toluidine blue, × 360) **Figure 7**—Abdominal aorta of a rat after 1 year on the CCT diet. Part of a large plaque, with obvious breakdown of the internal elastic lamina and severe medial damage. The endothelial covering is continuous and studded with adherent cells. Note the mitosis (*arrow*). (Epon section, toluidine blue, ×500)

25-fold peak at 3–6 weeks, followed by a slow decline; at 12–20 weeks the count was still almost 10 times normal. In CCT rats, there was a continuous rise to values about 50 times normal.

Electron Microscopy

The endothelium contained lipid droplets in increasing numbers with the duration of hypercholesterolemia; they were round or oval, with their long axis perpendicular to the luminal surface and often protruding above it (Figure 15). Myelin figures arising from the surface were common; some were very complex and electron-dense (Figure 15).

Ultrathin sections confirmed the presence of mononuclear cells attached to the endothelium (Figure 16). Most of these were identifiable as monocytes/macrophages. A minority had a round nucleus, no lyso-

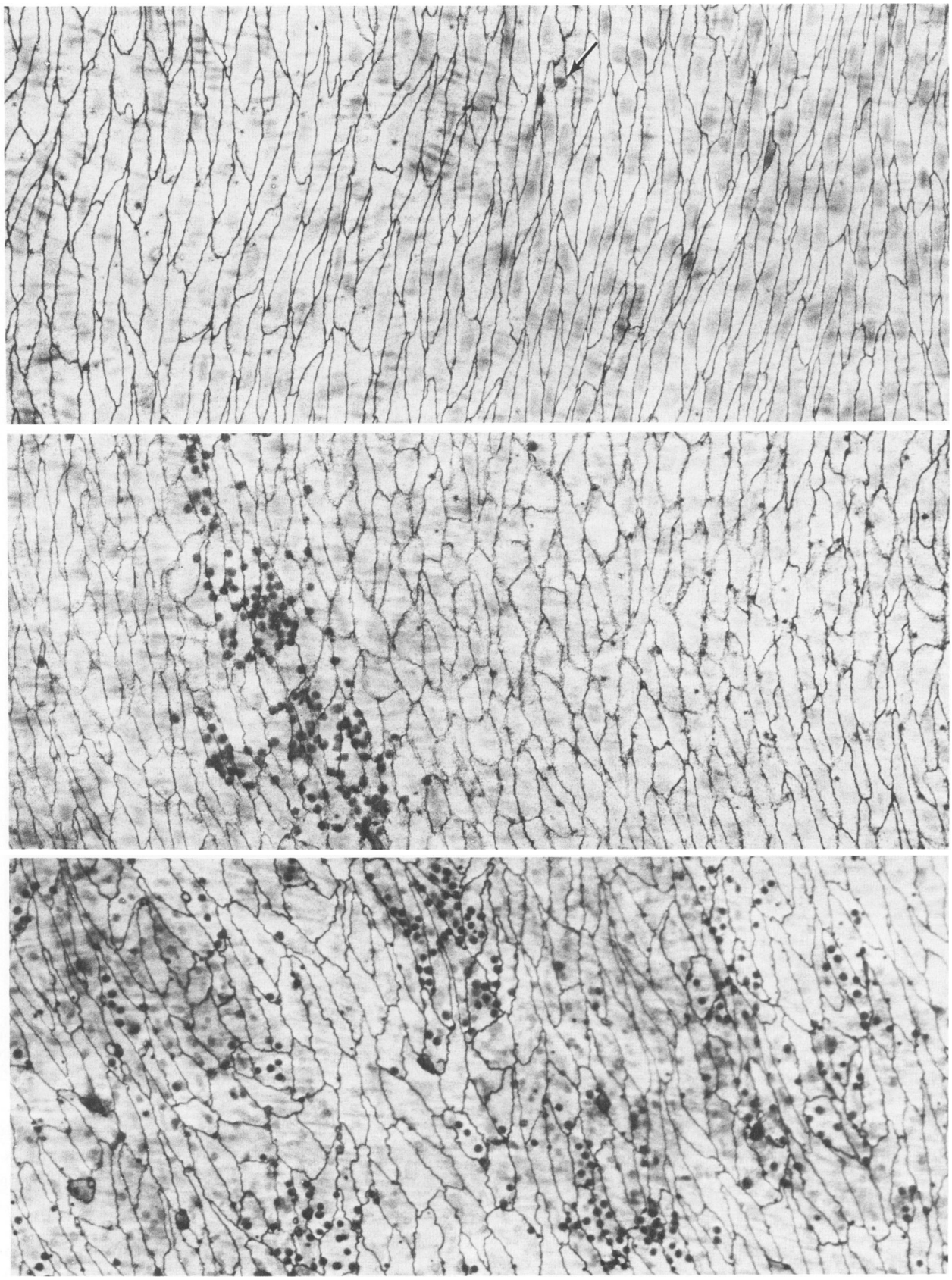


Figure 8—Control rat. Aortic intima viewed *en face* after staining with $AgNO_3$ and hematoxylin. The direction of flow is from top to bottom. The endothelial pattern is normal; a single mononuclear cell is attached (*arrow*). In this preparation the endothelial nuclei are barely visible; here and there the hematoxylin has stained the nuclei of superficial medial cells, oriented at right angles to the axis of the vessel. (×260)

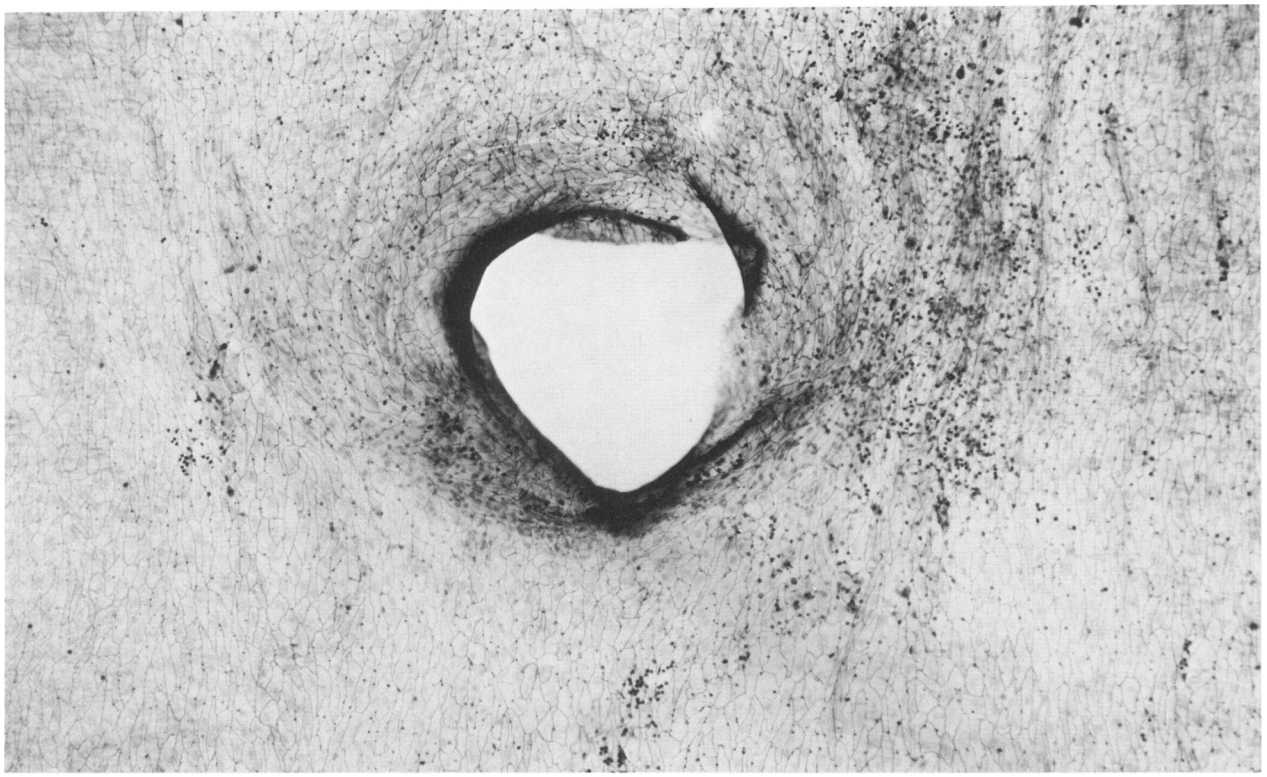


Figure 11—Aorta of a rat after 1 year on the CCT diet. Surface staining with $AgNO_3$ and hematoxylin. The direction of flow is from top to bottom. Ostium of an aortic branch. As in Figure 8, the dark dots correspond to cells attached to the surface; the peppering of gray dots represents cells that have migrated beneath the endothelium. There is no loss of endothelial cells. (×100)

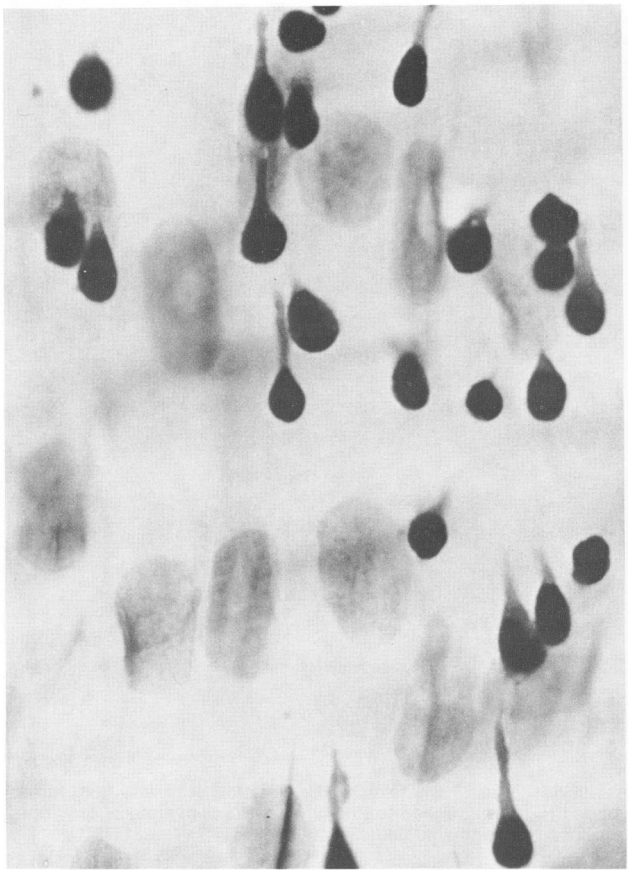
somes, many free ribosomes, and were classified as lymphocytes. Transendothelial passage of these cell types (referred to hereafter as *emigration*) was observed (Figures 16 and 17). The surface of the endothelium was studied beneath 70 adherent cells; in 49 cases, no abnormality was visible; in 10 cases, one or more myelin figures were present beneath the adherent cell, and in 5 others a myelin figure was seen in the immediate neighborhood (Figure 18); in 6 cases the endothelial area beneath and around the adherent cell was studded with tiny projections (Figure 16). Myelin figures were also found occasionally within phagosomes in adherent macrophages (Figure 19). Platelets were seen far less frequently than adherent mononuclear cells; their number increased with time on the hypercholesterolemic diet. They were attached to the endothelial surface as well as to adherent mononuclear cells. In early stages they were usually single and not degranulated; later they were present also in small clusters (Figure 20), and occasionally they were degranulated. In two instances a basophil was found, adhering to endothelium of normal appearance.

After 7–11 weeks on the diet, the macrophages beneath the endothelium acquired lipid droplets and eventually became foam cells (Figure 21). Such clusters of foam cells beneath an intact endothelium will be referred to hereafter as *fatty streaks*. At later stages the intimal lesions increased in size and complexity to form raised *plaques*; these were always covered by a continuous endothelial layer and contained two types of fat-laden cells as shown by 1-μ histologic sections (Figure 22). The extracellular space contained cholesterol crystals, basement membrane-like material, collagen fibrils, small osmiophilic deposits, and ring-shaped calcifications. Mitoses of smooth muscle cells were occasionally seen in the subintimal region; no mitoses were found in controls.

Discussion

We have shown that in rats fed diets that induce hypercholesterolemia two concomitant changes develop: a 5–20-fold increase in total plasma cholesterol and a 4–50-fold increase in the number of mononuclear

Figure 9—Aortic intima of a rat after 8 weeks on the CCT diet. Surface staining with $AgNO_3$ and hematoxyiin. The direction of flow is from top to bottom. Adherent mononuclear cells form an elongated cluster. There is no loss of endothelial cells. (×260) **Figure 10**—Aortic intima of a rat after 1 year on the CCT diet. Surface staining with $AgNO_3$ and hematoxylin. The direction of flow is from top to bottom. Adherent cells correspond to the darker dots; the gray dots correspond to the nuclei of cells that migrated beneath the endothelium. There is no loss of endothelial cells. (×260)



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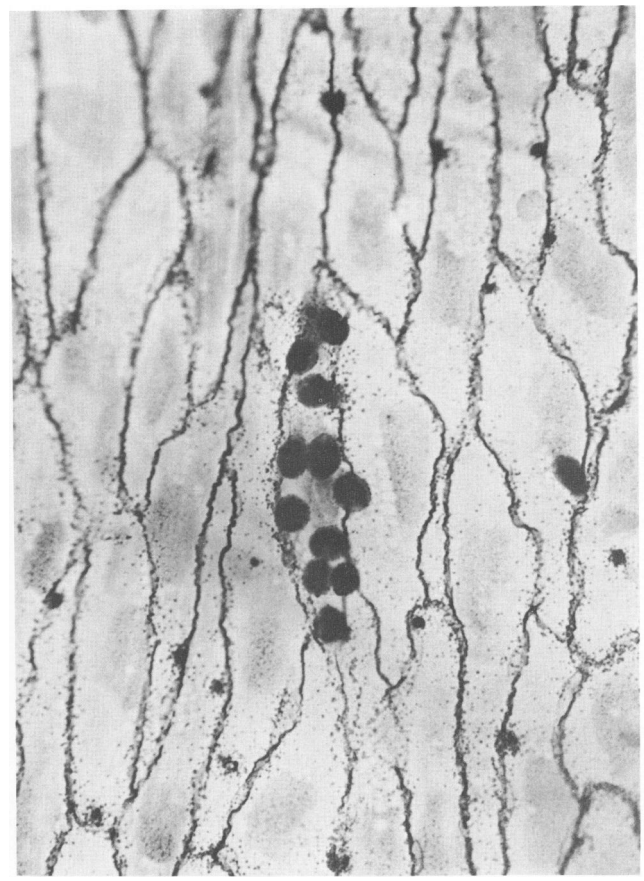

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Figure 12—Aorta of a rat after 11 weeks on the CCT diet. View *en face* after surface staining with hematoxylin only; direction of flow from top to bottom. The large, pale, oval nuclei correspond to endothelial cells; note the dark adherent mononuclear cells, with a tail pointing upstream. (×1250) **Figure 13**—Aorta of a rat after 1 year on the CCT diet; surface staining with $AgNO_3$ and hematoxylin. The direction of flow is from top to bottom. Eleven mononuclear cells are attached to the body and margins of a single endothelial cell. (×800)

cells adhering to the aortic intima. Cell adhesion and hypercholesterolemia remained elevated for the entire period of observation (1 year). Comparison of the two sets of data (Figures 1 and 14) shows that the two phenomena, once established, do not vary in parallel fashion. In part, this may be a sampling problem due to the focal nature of cell adhesion. However, it should be kept in mind that our cell counts apply only to *cells attached to the surface*. Since adhesion is followed by emigration, the number of surface cells does not necessarily reflect the dynamics of the entire adhesion–emigration phenomenon.

After they have settled in the subendothelium, the monocytes/macrophages tend to become loaded with lipid droplets and thus give rise to small lesions—flat or slightly raised—which can be equated to fatty streaks. How the lipid reaches the macrophages across an intact endothelium is not clear. It may be relevant to recall that hypercholesterolemia, in rabbits, causes a patchy increase in endothelial permeability not accompanied by endothelial injury visible by electron microscopy.[38,39] It is possible that the lipid may reach the macrophages after having been carried across the endothelium by "transcytosis."[40]

Some of these tiny fatty streaks in the CCT rats (but not in the CC rats) eventually became atherosclerotic plaques, comparable in type and location to those that others have obtained in similar rat mod-

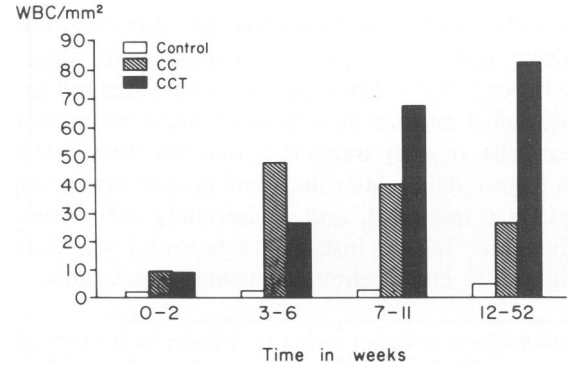
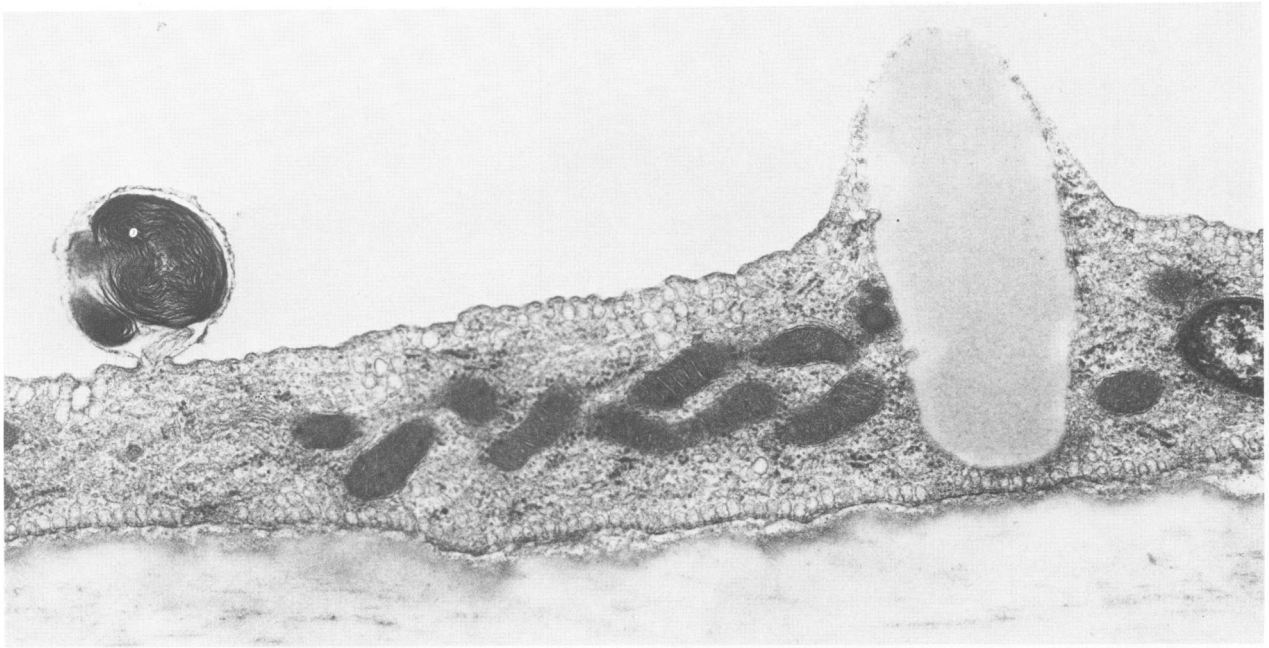


Figure 14—Quantitation of adherent cells per square millimeter on the aortic intima of control and hyperlipidemic rats. Number of square millimeters counted: 30–60 for each bar.



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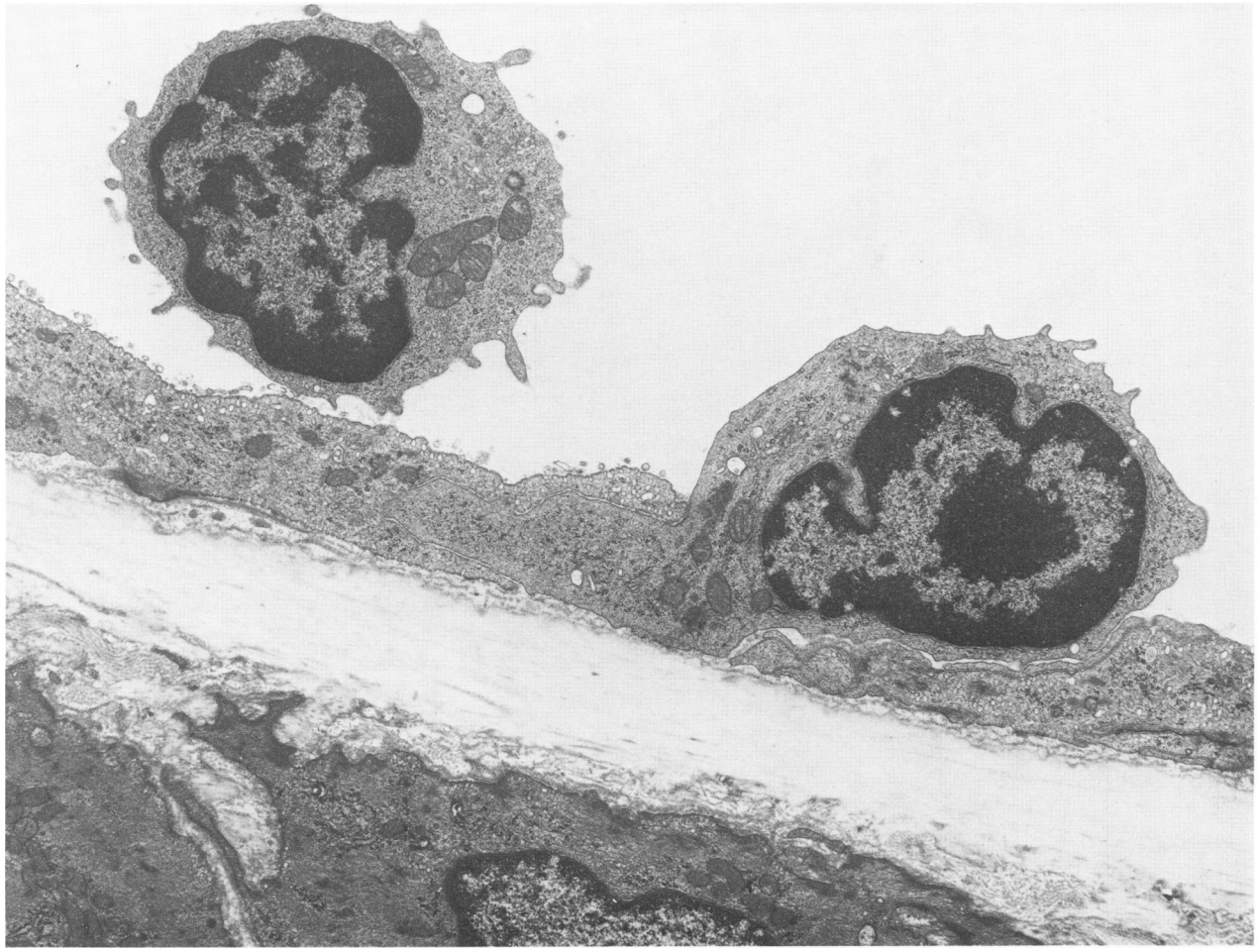

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Figure 15 — Aortic endothelium of a rat after 13 weeks on the CCT diet. *Left*: electron-dense myelin figure arising from the endothelial surface. *Right*: lipid droplet causing the endothelial surface to bulge. Notice the extreme attenuation of the endothelial cytoplasm above it. (× 27,500)
Figure 16 — Aortic endothelium of a rat after 11 weeks on the CC diet. *Left*: adherent mononuclear cell. Notice the fine projections arising from the endothelium beneath it. *Right*: another mononuclear cell (probably a macrophage) performing diapedesis. (× 11,400)

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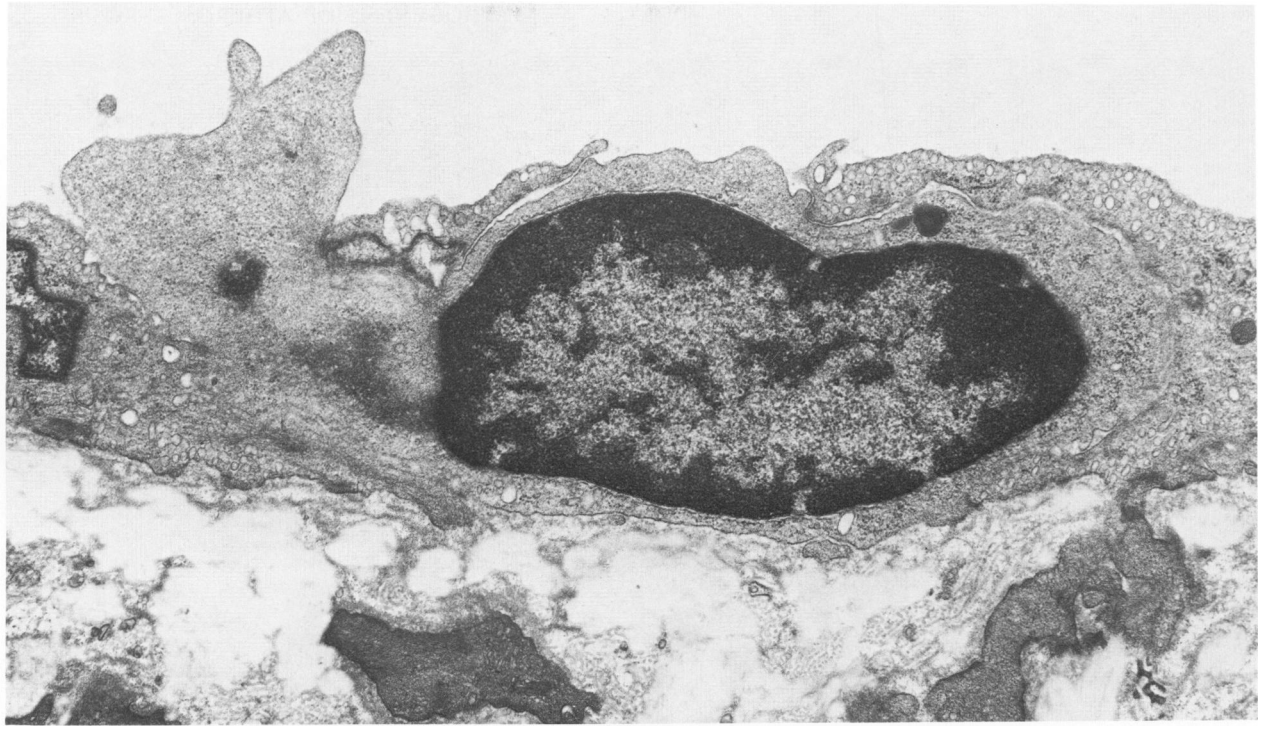

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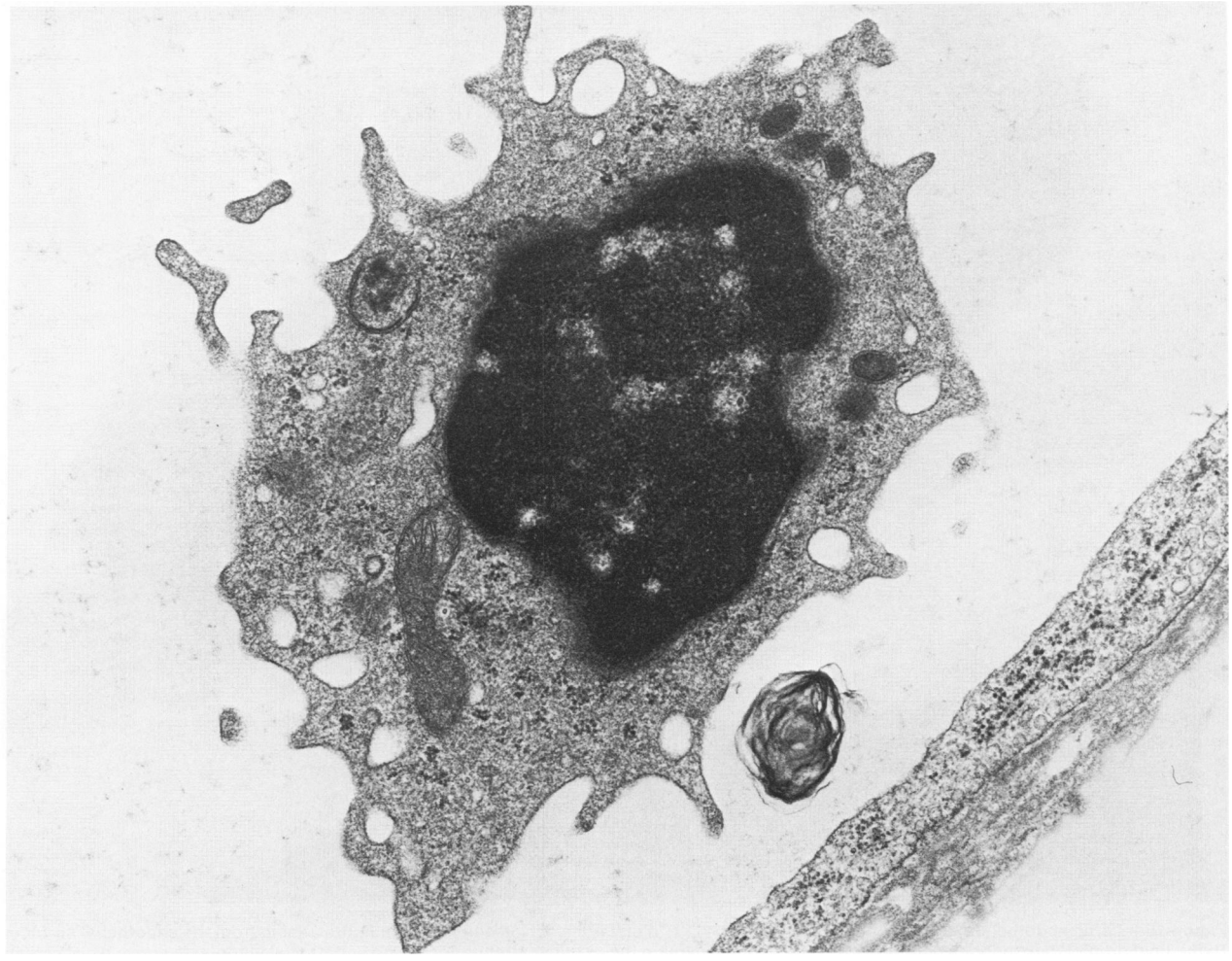

Figure 17 — Aortic endothelium of a rat after 11 weeks on the CC diet. A mononuclear cell is performing diapedesis. (× 18,200) **Figure 18** — Aortic intima of a rat after 11 weeks on the CCT diet. Grazing section across an adherent macrophage. Notice the rounded, electron-dense myelin figure that appears to lie between the macrophage and the endothelial surface. (× 30,000)

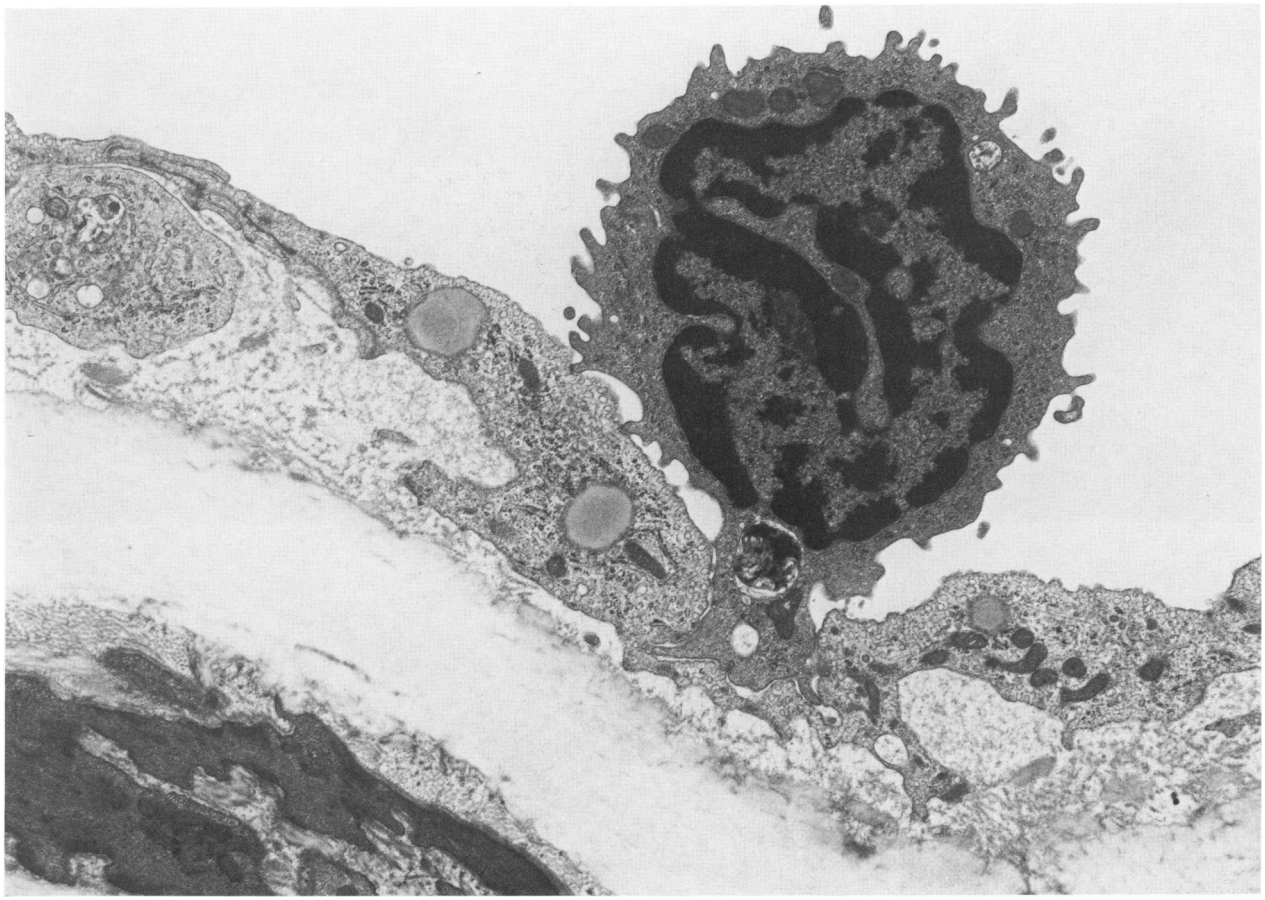

Figure 19—Aortic intima of a rat after 13 weeks on the CC diet. A macrophage is initiating diapedesis; the portion that is protruding within the endothelium contains an engulfed myelin figure. Notice the intraendothelial lipid droplets and part of a subendothelial cell at the left. (×11,250)

els.[6,41-44] By electron microscopy these plaques showed the basic features seen in human atherosclerosis (including smooth muscle cells and calcification). It is therefore reasonable to conclude that mononuclear cell adhesion, in the rat, is an early step in the pathogenesis of the atherosclerotic plaque. During the period of examination (1 week to 1 year) patches bare of endothelium did not occur. We therefore conclude that intimal denudation, in this model, is not a necessary step in the development of a plaque.

We cannot explain why cell adhesion and emigration occurred with both diets, whereas plaques appeared only in CCT rats. The difference may be very significant, in that it could provide a model for studying the progression from fatty streak to plaque. Further studies are needed for determination of whether the different behavior of the two groups is related to the different response of plasma lipoproteins (Figure 3) or to differences in the quality and quantity of the mononuclear cell response (Figure 14).

These findings raise a number of questions that are relevant to the pathogenesis of atherosclerosis in general:

1) *Which cell types participate in adhesion and/or emigration?*

About 90% of the adhering cells, as defined by their phagocytic properties (tested with carbon black), by the presence of nonspecific esterase activity, and by ultrastructure, were macrophages; some (in the order of 10%) appeared to be lymphocytes of a subspecies yet to be determined. Platelets were few; their numbers increased in the more advanced stages. Some degree of platelet adhesion should be expected, because a quantitative study of platelet adhesion in hypercholesterolemic monkeys has shown a rise as early as 10 days.[45] With regard to red blood cells, focal adherence was observed by others in arteries of "normal" animals[23] and remains unexplained. The chance finding of two adherent basophils in Epon sections is worth noting, in view of the extremely low number of circulating basophils in the rat.[46] Basophils (or mast cells) have been found in atherosclerotic lesions in man[1,47] and green monkeys.[25]

The types of cells participating in adhesion and emigration in hypercholesterolemic rats are the same that tend to adhere and emigrate (to a much lesser degree)

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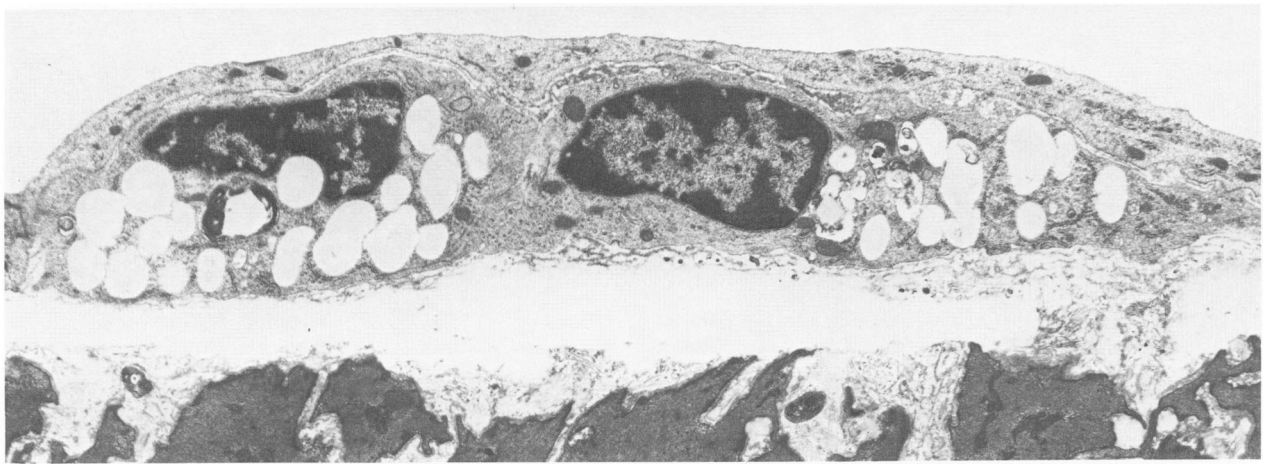

Figure 20 — Aortic intima of a rat after 11 weeks on the CC diet. Four mononuclear cells are attached to the surface; the third from the left is in the process of diapedesis between two endothelial cells. Notice the cluster of platelets at the top right, some showing early degranulation. (×4750) **Figure 21** — Aortic intima of a rat after 8 weeks on the CCT diet. Two lipid-laden macrophages are lying below the endothelium. This small lesion may be interpreted as a rudimentary fatty streak. (×6250)

under normal conditions.[1-4] Clearly, arterial endothelium is distinguished by the ability to induce selective adhesion and emigration of mononuclear cells, a property somewhat reminiscent of the "high endothelial venules" of lymphatic organs, which selectively trap lymphocytes.[48] In the microcirculation, acute inflammatory stimuli induce a preferential emigration of neutrophils and chronic stimuli induce emigration of mononuclear cells. Thus, the early phase of atherosclerosis can be said to overlap with the chronic inflammatory process.[1,2]

2) *What causes cell adhesion*?

The only clear-cut fact is that *mononuclear cell adhesion (in our model) occurs on endothelial cells* and not on denuded areas; thus, the mechanism should depend on a surface change of the cells involved, on a chemotactic effect,[49] or on a combination of both. Surface abnormalities of the endothelium in hyperlipidemic animals could be expected, since lipoproteins[50,51] and hypercholesterolemia can cause structural and/or functional endothelial changes.[38,39,52,53] In fact, myelin figures arising from the surface were common, especially in areas of cell adhesion: they were present immediately beneath 20% of the adherent cells, very close to another 7%, and even inside some macrophages (Figure 19), suggesting that the macrophage might have picked a myelin figure from the endothelial surface. The true incidence of myelin figures was certainly higher than estimated above, because small myelin figures are easily missed by the plane of section. Myelin figures of the density and complexity shown in Figures 15 and 18 cannot be attributed to fixation artifact; we have seen identical ones on the endothelium of rat aortas submitted to increased shear stress, presumably as an expression of nonlethal injury of the cell surface.[54] In a scanning electron microscopic study on CCT rats, Clowes et al[7] found small, smooth, spherical particles 0.5–1.5 μ in

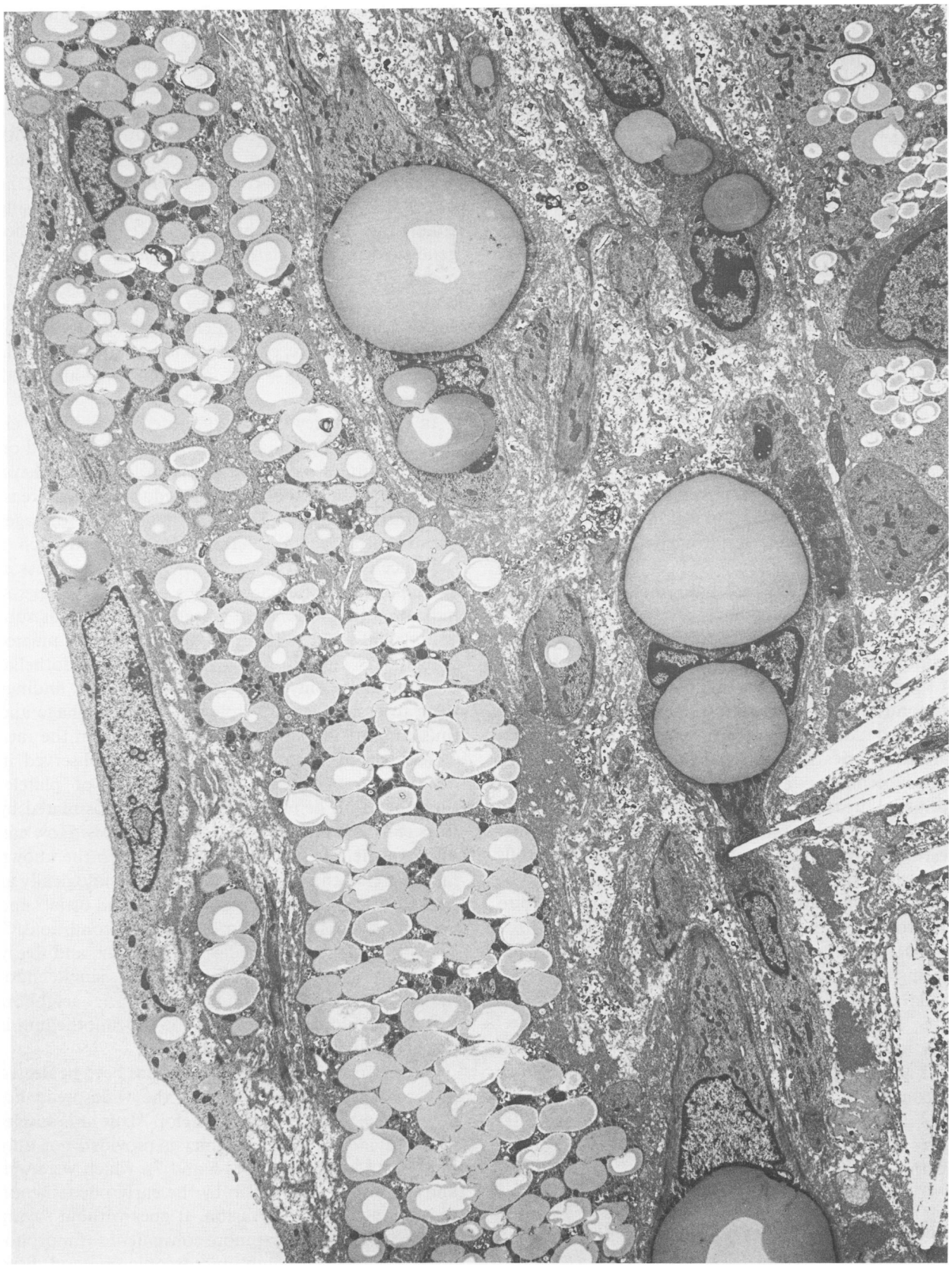

Figure 22—Aortic intima of a rat after 1 year on the CCT diet. Below intact endothelium (containing lipid droplets) lie macrophages laden with many small lipid droplets, smooth muscle cells containing 2–4 large lipid droplets, and crystals of cholesterol. The extracellular space contains basement-membrane-like material, collagen fibers, and small osmiophilic deposits, probably lipid. (×3750)

diameter on the endothelium and between adherent cells (their Figure 5) which were interpreted as "possible lipid remnants." Their aspect coincides closely with our myelin figures, as illustrated in our Figure 15. Similar structures appear in the electron micrographs of Gerrity et al.[11,14,15]

These data suggest that pathologic changes of the endothelial surface may contribute to attract mononuclear cells; we cannot rule out, however, that some surface changes may be caused by the presence of adherent cells.

A second mechanism of adhesion could depend on a ligand effect of circulating lipoproteins. Receptors for lipoproteins are present on the endothelium[52] as well as on macrophages and lymphocytes.[55-58] Conceivably, one or more types of lipoprotein molecules could act as bridges between the endothelium and mononuclear cells. Under normal circumstances, these bridges would be few and therefore relatively ineffective (this would explain the rare occurrence of mononuclear adhesion in normal animals); in hyperlipidemia the concentration of circulating ligands would be increased, resulting in more effective bonding.

A third possibility is that young, regenerating endothelial cells are somehow more sticky for mononuclear cells. There is much evidence to support this hypothesis. When coronary arteries undergo a burst of growth (as during the enlargement of collaterals) mononuclear cells adhere to their intima.[59] Mononuclear cell adhesion has also been reported on rat carotid arteries during repair after denudation.[7] In our experiments, adhesion occurred selectively in periostial areas (Figure 11), which are well known to have a high cell turnover.[60] These observations suggest that the peculiar clustering of mononuclear cells on a single endothelial cell (Figure 13) may also be due to the phase of that particular cell with regard to the growth cycle. It is not clear whether the *number* of LDL receptors is increased on the surface of regenerating cells (the *turnover* of LDL receptors, in endothelial cultures, decreases at confluence and increases after "wounding").[61]

A fourth possibility is that the mononuclear cells stick preferentially to dying cells. We are inclined to discount this explanation, because the endothelial cells beneath adhering monocytes or lymphocytes never show ultrastructural features suggestive of cell death.

Significance of the Macrophage-Lymphocyte Emigration Phenomenon

It should be emphasized again that the emigration of white blood cells into the arterial intima has been observed countless times.[5-25] The purpose of our study was to reexamine it sequentially in relation to cholesterolemia and to endothelial injury, and to analyze its role in the pathogenesis of the plaque. Our findings suggest that (in our model at least) plaque formation *begins* with mononuclear cell emigration, not with endothelial denudation followed by platelet intervention.[34] In this regard it is interesting that in the oft-quoted paper of Ross and Harker,[34] proposing endothelial damage as the primary event in the pathogenesis of atherosclerosis, endothelial injury was measured and illustrated only in monkeys that had been on a hyperlipidemic diet *for 1 year*; and the single scanning electron microscopic illustration of endothelial damage, pointing to two small endothelial lesions, also shows—with no comment—about 32 adherent cells.

In a later review[62] Ross proposed a modified version of the "endothelial injury" hypothesis, based on further studies of hypercholesterolemic monkeys; lesions were said to begin as deendothelialized areas, on which not only platelets but also macrophages became attached. We have never observed such a sequence of events in our model.[35] Still later, Ross et al[63] reported preliminary studies on pigtail monkeys, indicating that lesions are present at 1 month as subendothelial foam cells; surface attachment of macrophages was seen after 3–4 months, and endothelial desquamation only after 5 months. These findings seem to assign an early role to the macrophage and thus support the validity of our findings in the rat.

The lack of endothelial denudation, observed in our model, necessarily implies a lack of platelet thrombosis on denuded surfaces, long postulated by Ross et al as an initial step in atherogenesis. How can we reconcile this lack of denudation with the known fact that endothelial cells slough off physiologically as a result of cell turnover, especially around ostia[60] and even more rapidly in hyperlipidemic conditions[64]? The answer lies in the studies of Schwartz, and Reidy and Schwartz, who showed that dying, single endothelial cells are "undermined" by their neighbors before they slough off, so that the subendothelium is not exposed to the blood.[65,66]

The rat model of atherosclerosis has been neglected in recent years, largely due to the widespread belief that the rat does not develop "true atherosclerosis." Evidence to the contrary is provided not only by the late lesions (Figures 6 and 7), which were well known,[5,41-44] but now also by the early phenomenon of mononuclear cell invasion. It goes without saying that the rat model is far more convenient for morphologic studies. The light-microscopic method used herein (study of the aorta *en face* after surface staining) is simpler than the Häutchen methods, preserves

the subendothelial structures, and allows a quick topographic overview of the whole vessel.

We may now propose an answer to the question raised at the opening of this paper: the tendency of mononuclear cells to invade the "normal" arterial intima, observed in many laboratory animals, represents "minimal arteriosclerosis."

Further work is needed on the molecular changes that hyperlipidemia induces on the surface of the cells involved (endothelium, macrophages, lymphocytes), and on the role of macrophages as well as of lymphocytes once they have settled onto and within the arterial intima.

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